Appendix I

Feasibility Demonstration Project for HTPS

[Note to the reader: The following text and chart were supplied by OSI Pharmaceuticals, Inc., in response to EPA's RFP on the demonstration feasibility study for high throughput pre-screening (HTPS). OSI Pharmaceuticals, Inc., was the only company to submit a response to the RFP and was awarded the contract for the demonstration project.]

OSI PHARMACEUTICALS, INC.
HIGH THROUGHPUT PRE-SCREENING RESEARCH PLAN

Executive Summary

Profiling of chemical entities has undergone a revolution in recent years with emerging rapid technologies measuring endocrine modulation. Endocrine modulation by chemicals dispersed into the environment can have serious consequences for wildlife and humans and has resulted in legislation included in the Safe Drinking Water and Food Quality Protection Acts requiring safety assessments of manufactured chemicals.

OSI Pharmaceuticals, Inc., has recognized the need for rapid chemical assessment tools and is developing a core, modular chemical profiling technology platform which builds from the existing strength of the organization and provides solutions to many of these issues.

The technology has focused on the generation of panels of reporter gene constructs which will provide profiling information regarding the potential for chemical compounds to activate the estrogen receptor (α and β), androgen receptor, and/or thyroid hormone receptor. A panel of highly sensitive, stably transfected human and/or rodent cell lines are available which, together with customized automation and an informatics/quantitative structure-activity relationship (QSAR) package, will enable the rapid generation of high quality and quantitative data sets. The approach provides for a near turn key system enabling the routine testing of active molecules from a variety of sources (pharmaceutical, chemical, agrochemical).

The attached research plan covers the core features of the program and a proposed initial panel of gene constructs and compound libraries which could be employed in validating the approach. Options to the core program include choices in cell lines, the scale and throughput capabilities of the automation, and the nature and make-up of the molecular markers used in profiling.

The key advantage of this initiative stems from its harnessing of the collective skills, experience, know-how and intellectual property (US 5,665,543; US 08/267,834; US 08/458,691; and WO 94/17208) of OSI Pharmaceuticals, Inc., and that cross-licensed from Xenometrix, Inc., to deliver a technology platform which we believe will be of great value to the pharmaceutical, chemical, and agrochemical industries. Implementation time of a staged program is short: (1) approximately 6 months for initial validation/demonstration studies on 50 - 100 compounds (a workplan is attached as page 2; (2) 3

months for compound acquisition and formatting for the full screening phase (proposed); and (3) 12 months for profiling of 20,000 compounds in ten assays (proposed). It should be emphasized that OSI Pharmaceuticals, Inc., will work extensively with the Environmental Protection Agency (EPA) to tailor the screening cell panels, robotics, and informatics programs to suit the regulating agency's needs.

Staged chemical profiling proposal

Stage 1: 6 month validation/demonstration study employing 50 B 100 chemical compounds (specified by EPA) designed to test the overall performance and sensitivity of the cell based assay systems. Cost $70K, including compound acquisition and plating up to $5K. We have sourced and priced the first 45 of an estimated 70 B 100 compounds to be tested and anticipate that the $70K project budget will be sufficient to procure the test compounds. Specialty compounds requiring contract synthesis are not anticipated.

1) Endocrine modulator assays (10 assays with controls)

- Estrogen receptor assay measuring both α and β isoforms in MCF-7 human breast cells measuring activation and inhibition, with and without endogenous cyp3A4 metabolism.
- Androgen receptor assay in MDA453 human breast cells measuring activation and inhibition, with and without endogenous cyp3A4 metabolism.
- Thyroid hormone receptor assay in MCF7 human breast and/or HeLa cervical carcinoma cells measuring activation and inhibition, with endogenous cyp3A4 metabolism.

(Proposed) Stage 2: 3 month compound acquisition phase in which ~ 1mg (or flick) of compound is dispensed into OSI Pharmaceuticals, Inc. supplied bar-coded vials and returned to OSI Pharmaceuticals, Inc., for inventory and formatting of master compound screening plates. Compounds can be supplied with structures (in electronic format) or anonymously. Alternatively OSI Pharmaceuticals has the capacity to source compound through standard suppliers and format the necessary screening plates directly.

(Proposed) Stage 3: 12 month high-throughput screening campaign of a maximum of 50,000 compounds at a rate of 38,000 assays/day or 300 compounds/day in 10 assays using a 5 point dose-response in duplicate. Data reduction and QC are handled on-line. QSAR modeling can be provided given structural information. Cost $3.9M.

Molecular markers

Steroid response element	Cell line	Steroid target
ERE -	MCF-7 (breast)	estrogen (α and β receptors)
ARE -	MDA-453 (breast)	androgen
TRE -	MCF-7 (breast)	thyroid hormone
	HeLa (cervical)	

ERE, ARE, and TRE are the DNA response elements which bind the corresponding activated receptor and modulate gene transcription. The fusion constructs contain 4 copies of the ERE, ARE or TRE fused to the HSV TK promoter (containing mutations to reduce basal activity) and luciferase reporter gene. Reporter constructs also contain selectable marker (SV-puro) which confers resistance of transfected cells to the antibiotic puromycin.

Cell lines and characteristics

MCF-7

The cell line MCF-7 was derived from a human breast carcinoma and will be used to evaluate potential compound modulation of the estrogen receptor (ER). The assay measures binding of the ER to the ERE DNA sequence, resulting in increased reporter gene expression (luciferase). It can detect agonists and antagonists of estrogen. The ERE DNA sequence does not bind significantly either the progesterone receptor, glucocorticoid receptor, androgen receptor, thyroid receptor, vitamin D receptor or retinoid receptors. MCF-7 contains both α and β ER isoforms.

MDA-MB-453

Human breast cancer cell line MDA-MB-453 will be used for measurement of androgen receptor modulation through the ARE construct. This line has high androgen receptor number and responds well to androgen stimulation. Androgen receptor, progesterone receptor, mineralocorticoid receptors and glucocorticoid receptors all can bind the ARE DNA sequence. However the MDA-MB-453 cell line exhibits very low level responses to estrogen, progesterone and glucocorticoid in comparison to the androgen receptor response, making this line ideal for these studies.

MCF-7/HeLa

Both the breast cell line MCF-7 and the HeLa cervical carcinoma cell line will be evaluated for thyroid hormone receptor modulation through the TRE construct. Both lines are commonly used to measure TRE activation and inhibition and the line showing the best response will be used for compound screening.. It should be noted that both thyroid hormone receptors (TR α, β) and retinoic acid receptors (RARs and RXRs) interact with the TRE construct, either as homodimers or as heterodimers. Both TR and RAR/RXRs serve as targets for compound modulation of endocrine and development functions. The proposed cell lines are suitable for detecting TR/RXR interaction.

Cyp3A4 metabolic activation

In situations where it is desirable to screen drug candidates in the presence of metabolic activation by the cytochrome P450 isozyme, CYP3A4, we propose using engineered MCF-7, MDA453 and HeLa cell lines constitutively expressing 3A4. This isozyme is responsible for metabolizing 70% of known drugs. The cyp3A4 cDNA is licensed through the National Cancer Institute (Dr. F. Gonzales).

Assay systems

Promoter sequences will be cloned upstream of the luciferase reporter gene from *P. pyralis*. The sensitive and quantitative reporter vector, pUV120 includes a bacterial origin of replication for propagation in *E. coli*. These vectors carry splicing and polyadenylation sequences for correct processing of luciferase mRNA transcribed by the target promoter, a polylinker sequence for insertion of foreign promoters 5' of the luciferase reporter, a termination signal 5' of the inserted promoter sequence that serves to prevent transcriptional read-through from upstream transcription units following integration in stable cell lines, and, finally, eucaryotic selectable markers (SV-puro) for positive selection of recipient cells following transfection. These vectors have been constructed and optimized by OSI Pharmaceuticals, Inc., and allow for sensitive detection of even weak promoter expression.

Electroporation is the preferred method for the generation of stable cell lines harboring linearly integrated reporter gene constructs. Briefly, reporter construct DNAs are linearized by restiction endonuclease digestion, the linear DNA is transferred into the recipient cells by electroporation and antibiotic (puromycin or neomycin) is added to select for those cells which stably integrate the construct DNA. Resistant cells clones are picked and analyzed for correct regulation of the integrated luciferase reporter construct and luciferase expression, where detergent extracts from transfected cells are incubated with luciferin, ATP, Mg2+ and DTT under standard conditions. Luciferase expression from the target promoter is further characterized for: (i) faithful integration of linear construct DNA by isolation of genomic DNA and Southern blot hybridization analysis; and (ii) correct inducible regulation of the integrated reporter construct.

Molecular and Cellular Biology

OSI Pharmaceuticals, Inc., has considerable strength in state-of-the-art molecular and cellular biology. This includes a highly trained team of molecular biologists and chemists, who are capable of rapidly identifying and isolating regulatory regions involved in the control of transcription of target genes, as well as constructing defined reporter vectors used to monitor even minimal changes in gene expression. These vector systems are stably integrated in the appropriate cellular background, to produce cell lines for high throughput screening. Expertise in transcription allows the multidisciplinary team to perform rapid mechanism of action studies to identify mechanism(s) of action the molecular target of a given compound on a molecular target rapidly and comprehensively. OSI Pharmaceuticals, Inc., has unparalleled cell culture facilities to address the importance of cell based assay systems which include 30 stand-alone tissue culture incubators, hot room facilities for large scale suspension culture, and 20 custom designed and developed tissue culture incubators incorporated into our robotic screening systems.

Screening technology

In order to facilitate the screening of large numbers of test samples, OSI Pharmaceuticals, Inc. has invested extensively in the development of proprietary robotics systems. This screening approach combines live genetically engineered cells and unique robotics, enabling the screening of greater than five million compounds annually.

The robotics systems handle every step in the assay procedure. The systems consist of fully automated tissue culture incubators, liquid handling / dispensing systems for the dilution and addition of test samples, and an array of robotics units for manipulation of each step of the assay loop. Robotic arm assemblies are employed to shuttle microplates through the assay cycle. Profiling assays culminate with a read-out from a 96-well luminometer. Data are captured automatically into a processing network that performs quality control (QC) analysis on each individual microplate and carries out a rapid data reduction to identify active compounds. Automation on this scale has proven to be essential for producing high quality data from cell based screens. In addition, it has provided a number or major advantages over other screening approaches:

(i) High-Throughput: The robotic system designed for cell panel profiling can comfortably evaluate 300 compounds per day, in a five point dose response against 10 assay targets (3 endocrine targets up and down; 2 targets with and without cyp3A4 metabolism). Duplicate determinations are averaged to yield a mean inhibition or stimulation of receptor activity. Additional positive and negative controls are included on each microtiter plate to provide clear functional plate pass/fail criteria. Potential compound cytotoxicity is minimized by using a short incubation time of between 12 and 24 hours. Compounds which do inhibit basal promoter activity by greater than 2 standard deviations will be further studied using a standard cytotoxicity assay measuring mitochondrial respiration (MTT) to derive a cytotoxicity IC50 value for that compound.

(ii) Accuracy: Automation has proven to be highly effective in removing protocol variation and in more accurately controlling and synchronizing procedures. With the current technology, cell-based high-throughput screens can be run with coefficients of variation of less than 15%.

(iii) Cost Effectiveness: Support for each robotics system requires only a three-person team. This includes the staff for all tissue culture, robotics maintenance, robotics operation, and rudimentary initial data analysis.

(iv) Compound Handling: Over the last several years, OSI has been successful in compiling an extensive library of screening samples. We have developed robotic systems which enable the rapid preparation of large compound libraries into a 96-well format suitable for compound screening. This approach offers the possibility of archiving sets of master compound plates for future screening needs. This robotic system is capable of supporting the compound needs of our four screening systems and handles our growing collection (including collaborators libraries) of more than 1.5 million synthetic organic compounds.

(v) Automation and Information Handling: The Informatics Technology Development group at OSI possesses expertise in the areas of database administration, application development, and data analysis. Currently, the staff consists of an Oracle certified database administrator, an Oracle certified master developer, an ISIS certified database administrator, a computational chemist experienced in data analysis, and personnel with expertise in the development of Internet/Web-based tools. The primary responsibility of the team is to provide services of information management and interpretation. Currently, we have developed an in-house version of Xenometrix's molecular toxicology database.

(vi) Quality Control Criteria and Validation of the Gene Profiling Panel: The proper construction of the profiling panel will be verified by several criteria: (1) the DNA constructs will be properly integrated within the cell line (unrearranged); (2) the reporter signal strength will be sufficient for robotic high-throughput screening for both agonist and antagonist activity; (3) the marker genes will respond appropriately using a series of compounds containing chemical classes which selectively induce expression of the individual target genes.

(vii) Report Format: Reports will contain data tables with compound ID number, compound structure (if available), raw data (luciferase units), EC_{2X} (agonist activity), IC_{50} (antagonist activity). Footnotes can include specifics with regards to assay conditions, limitations and/or difficulties associated with a given data point.

(viii) Success in applying high-throughput screening to the discovery of therapeutically useful compounds: OSI Pharmaceuticals, Inc., has successfully utilized with HTS robotic screening technology in the area of cancer with compound in Phase I trials (in collaboration with Pfizer), and with compounds active in animals identified in the therapeutic areas of inflammation, cardiovascular disease, diabetes, anti-viral, prescription cosmetics and anemia. Last year, over 7 million compound equivalents were screened in multiple therapeutic programs.

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